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Inhibition of Semicarbazide-sensitive Amine Oxidase Reduces Atherosclerosis in Apolipoprotein E-deficient Mice

Running title: Inhibition of SSAO Reduces Atherosclerosis

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Key words: vascular adhesion protein-1, semicarbazide-sensitive amine oxidase, atherosclerosis, coronary artery disease

Abbreviations

AGEs = advanced glycated end-products; ALEs = advanced lipoxidation end-products; VAP-1= vascular adhesion protein-1; SSAO = semicarbazide-sensitive amine oxidase; CAD = coronary artery disease; ApoE = apolipoprotein E; SMC = smooth muscle cells; ROS = reactive oxygen species; LDL = low-density lipoprotein; H_2O_2 = hydrogen peroxide; advanced glycation end-products; LOX = lysyl oxidase; S1P = sphingosine-1-phosphate; OGTT = oral 75-g glucose tolerance test; LDL-C = low-density lipoprotein cholesterol; HDL-C = high-density lipoprotein cholesterol; ALT = alanine transaminase; AST = aspartate aminotransferase; MCP-1 = monocyte chemoattractant protein-1; TLR-4 = Toll-like receptor-4; RAGE = receptor for advanced glycation end-products; MMP-9 = matrix metalloproteinase-9; VCAM-1 = vascular cell adhesion molecule-1; ICAM-1 = intercellular adhesion molecule-1; PCNA = proliferating cell nuclear antigen; TNF-α = tumor necrosis factor alpha; LOX-1 = lectin-like oxidized low-density lipoprotein receptor-1; HUVEC = human umbilical vein endothelial cells; ECM = endothelial Cell Medium; HUVEC hSSAO/VAP-1 cells = HUVEC overexpressing VAP-1/SSAO; A7r5 hSSAO/VAP-1 cells = A7r5 cells overexpressing VAP-1/SSAO; LPS = lipopolysaccharide; H_2DCF-DA = dichlorofluorescein diacetate; PDGF = platelet-derived growth factor; BMI = body mass index

Background

Vascular adhesion protein-1 (VAP-1) participates in inflammation and has semicarbazide-sensitive amine oxidase (SSAO) activity. However, the effect of VAP-1/SSAO inhibition on atherosclerosis remains controversial.

Translational Significance

- Plasma VAP-1/SSAO concentrations are positively associated the presence and the extent of coronary artery disease in humans.
- PXS-4728A, a specific VAP-1/SSAO inhibitor, can reduce atherosclerosis in cholesterol-fed ApoE-deficient mice, through suppression of many key steps for atherosclerosis, including reactive oxygen species generation, endothelial dysfunction, adhesion and transmigration of monocytes, recruitment and activation of macrophages, as well as migration and proliferation of SMC.
- VAP-1/SSAO inhibition is a potential treatment for atherosclerotic cardiovascular disease.

Abstract

Inflammation, oxidative stress and formation of advanced glycated/lipoxidation end-products (AGEs/ALEs) are important for atherosclerosis. Vascular adhesion

protein-1 (VAP-1) participates in inflammation and has semicarbazide-sensitive amine oxidase (SSAO) activity, which catalyzes oxidative deamination to produce hydrogen peroxide and aldehydes, leading to generation of AGEs/ALEs. However, the effect of VAP-1/SSAO inhibition on atherosclerosis remains controversial, and no studies used coronary angiography to evaluate if plasma VAP-1/SSAO is a biomarker for coronary artery disease (CAD). Here, we examined if plasma VAP-1/SSAO is a biomarker for CAD diagnosed by coronary angiography in humans and investigated the effect of VAP-1/SSAO inhibition by a specific inhibitor PXS-4728A on atherosclerosis in cell and animal models. In the study, VAP-1/SSAO expression was increased in plaques in humans and apolipoprotein E (ApoE)-deficient mice, and colocalized with vascular endothelial cells and smooth muscle cells (SMC). Patients with CAD had higher plasma VAP-1/SSAO than those without CAD. Plasma VAP-1/SSAO was positively associated with the extent of CAD. In ApoE-deficient mice, VAP-1/SSAO inhibition reduced atheroma and decreased oxidative stress. VAP-1/SSAO inhibition attenuated the expression of adhesion molecules, chemoattractant proteins, and pro-inflammatory cytokines in aorta, and suppressed monocyte adhesion and transmigration across human umbilical vein endothelial cells. Consequently, the expression of markers for macrophage recruitment and activation in plaques was decreased by VAP-1/SSAO inhibition. Besides, VAP-1/SSAO inhibition suppressed proliferation and migration of

A7r5 SMC. Our data suggest plasma VAP-1/SSAO is a novel biomarker for the presence and extent of CAD in humans. VAP-1/SSAO inhibition by PXS-4728A is a potential treatment for atherosclerosis.

Introduction

Atherosclerosis is the leading cause of death in developed countries.[1,2] The World Health Organization (WHO) estimated that 17.5 million people died from cardiovascular diseases, representing 31% of all global deaths.[3] Inflammation has been shown to be another important mechanism for the progression of atherosclerosis.[4,5] Circulating leukocytes, especially monocytes, transmigrate from the vasculature to the inflammation site and are activated. Various chemokines, cytokines, enzymes, and reactive oxygen species (ROS) are produced and secreted, which may modify low-density lipoprotein (LDL), propagate inflammation, and result in atherosclerosis. Therefore, therapeutic agents able to suppress inflammation and reduce ROS in the arterial wall are of great potential as new anti-atherosclerotic drugs.

Vascular adhesion protein-1 (VAP-1) is an endothelial adhesion molecule involved in leukocyte rolling, adhesion and transmigration into inflammatory sites.[6] VAP-1 is expressed in endothelial cells, smooth muscle cells (SMC), and adipocytes.[7,8] Different from other adhesion molecules, VAP-1 has an enzymatic function, called semicarbazide-sensitive amine oxidase (SSAO) [EC 1.4.3.21], which catalyzes the oxidative deamination of primary amines into aldehydes (e.g., formaldehyde, methylglyoxal), hydrogen peroxide (H_2O_2), and ammonia.[9,10] Under

enhanced oxidative stress, aldehydes can cross-link proteins and result in the generation of advanced glycation/lipoxidation end-products (AGEs/ALEs), both of which can induce endothelial injury and promote monocyte activation.[11-14] H_2O_2 is a source of ROS, which can modify LDL in the arterial wall. Besides, both hydrogen peroxide and ammonia are cytotoxic to vascular cells at high concentrations. In other words, all these end-products of VAP-1/SSAO can result in the development of atherosclerosis. Indeed, chronic administration of methylamine, a substrate of SSAO, resulted in increased atheroma area in mice.[15]

On the other hand, there is a soluble form of VAP-1 that can be measured in plasma. Soluble VAP-1 is derived from the transmembranous form of VAP-1 by proteolytic cleavage.[16-19] Soluble VAP-1 is upregulated and released in certain inflammatory disorders, such as diabetes[20,21] and hepatitis[16] among others. In humans, serum VAP-1 is associated with atherosclerosis in carotid arteries[22,23] and the incidence of major cardiovascular events[24]. Moreover, serum VAP-1 can predict cardiovascular mortality in both type 2 diabetic subjects[25] and general population[24]. Taken together, findings from these reports suggest that VAP-1/SSAO is involved in atherogenesis and can be a novel target for the treatment of atherosclerosis.

So far, there are limited studies that investigate the effects of VAP-1/SSAO inhibition on atherosclerosis.[26-28] Two of them used semicarbazide to inhibit

VAP-1/SSAO activity in LDL receptor knock-out mice, with conflicting results. In one report, semicarbazide aggravated atherosclerotic lesions.[27] However, semicarbazide could stabilize the established atherosclerotic lesions in another report.[26] Off-target effects of semicarbazide may be one of the reasons for the inconsistent findings, since semicarbazide is not a specific inhibitor for VAP-1/SSAO and also inhibits enzymes which are involved in atherogenesis, such as lysyl oxidase (LOX) and sphingosine-1-phosphate (S1P) lyase.[29-34] Besides, another study using a small molecule VAP-1 inhibitor (LJP1586) for 4 weeks did not find a significant regression of atherosclerosis.[28] However, a 4-week treatment length may not be long enough so that it may underestimate the treatment efficacy.

PXS-4728A is a highly specific inhibitor of VAP-1/SSAO, and does not have any significant off-target effects when tested against other amine oxidases or over 100 different macromolecular targets.[35] In this study, we investigated whether VAP-1/SSAO inhibition reduced atherosclerosis using this specific inhibitor in apolipoprotein E (ApoE)-deficient mice for 15 weeks. Then, we explored the potential mechanisms in cell models. Although there were some studies which investigated the role of plasma VAP-1/SSAO as a biomarker for atherosclerosis,[21,22,24,36-38] none of them used coronary angiography to confirm the presence and the extent of coronary artery disease (CAD). Therefore, we conducted a human study, which examined the

association of plasma VAP-1/SSAO to the presence and the extent of CAD diagnosed by coronary angiography.

Materials and methods

- **Human study**

Patients

From 2008 to 2009, we conducted a cross-sectional study at National Taiwan University Hospital.[39] Subjects who were suspected to have CAD based on positive results of noninvasive stress tests and who received selective coronary angiography were invited to participate in this study. All subjects were evaluated by cardiologists. They were asked to answer questionnaires and receive physical examinations and blood tests, with the aid of trained nurses. The choice of noninvasive stress tests was determined by the attending physician, such as treadmill electrocardiography and thallium perfusion scanning. None of the study subjects took medications which may affect the activity of serum SSAO or VAP-1.[40] Written informed consent was obtained from each participant. The study protocol was approved by the institutional review board and the study was performed in accordance with the Declaration of Helsinki of Human Research.

Measurements and assays

Plasma samples were collected from each participant after overnight fasting and then stored at -80℃ before the measurement of VAP-1/ SSAO concentration. A standard oral 75-g glucose tolerance test (OGTT) was performed to measure 2-h postprandial plasma glucose. Plasma glucose and lipid profiles were measured with an automatic analyzer (Toshiba TBA 120FR, Toshiba Medical Systems Co., Ltd., Tokyo, Japan). Plasma VAP-1/SSAO concentration was measured by a quantitative sandwich enzyme immunoassay (R&D Systems, Minneapolis, USA). The assay utilized a specific monoclonal antibody against VAP-1 as a capturer on a microplate. Detection of bound VAP-1 was performed using a different polyclonal antibody specific for VAP-1 conjugated to horseradish peroxidase. A substrate solution was added and the intensity of the color was measured, which was in proportion to the amount of plasma VAP-1 bound in the initial step. Plasma VAP-1/SSAO concentration was quantified on the basis of a reference sample of highly purified recombinant human plasma VAP-1/SSAO. The intra-assay and inter-assay coefficients of variation were 1.90% and 3.58%, respectively.

Definition

CAD was determined by coronary angiography and defined as the presence of at least one stenosis of ≥50% diameter in any of the major epicardial coronary arteries. [41] The extent of CAD was assessed based on the number of atherosclerotic segments and

the number of major epicardial coronary arteries with ≥50% stenosis. Diabetes was diagnosed if fasting plasma glucose ≥126 mg/dL, 2-h postprandial plasma glucose ≥200 mg/dL, hemoglobin A1c ≥6.5% or the patient used pharmacological treatment for diabetes.[42] Hypertension was defined if systolic blood pressure ≥140 mmHg, diastolic blood pressure ≥90 mmHg, or the patient used antihypertensive drugs.[43]

- **In vivo and in vitro study**

Animal groups and treatment

Male 8-week ApoE-deficient mice were purchased from Animal Resources Centre (Canning Vale, WA, Australia) and kept under a C57/BL6 background. All procedures were performed in accordance with the local institutional guidelines for animal care of the National Taiwan University and complied with the "Guide for the Care and Use of Laboratory Animals" NIH publication No. 86-23, revised 2011. The protocol was approved by the National Taiwan University College of Medicine and College of Public Health Institutional Animal Care and Use Committee (IACUC NO: 20130274). Animals were randomly distributed into five groups: Group I (Control), fed with standard chow diet (n=12); Group II (Cholesterol Diet), fed with cholesterol-enriched diet (Purina Mills, Inc., USA) containing 21% fat (12% saturated fat) and 0.15% cholesterol for 15 weeks (n=12); Group III (Cholesterol

Diet/PXS-4728A, the prevention group), fed with cholesterol-enriched diet and PXS-4728A (10 mg/kg/day in 50 µl of 5 mM Na2HPO4 solution, Pharmaxis, Australia) orally for 15 weeks (n=12); Group IV (Cholesterol Diet/PXS-4728A 7W, the treatment group), fed with cholesterol-enriched diet for 15 weeks and PXS-4728A (10 mg/kg/day) orally from 9-15 weeks (n=12); Group V (Cholesterol Diet/atorvastatin, the positive therapeutic control group), fed with cholesterol-enriched diet and atorvastatin (2.5 mg/kg/day, Pfizer, USA) orally for 15 weeks (n=12). Fasting blood samples were collected one day before the experiment commencement (0 time) and at the end of the experiment (after 15 weeks) for measurement of plasma triglycerides, total cholesterol, low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C), glucose, creatinine, alanine transaminase (ALT), and aspartate aminotransferase (AST) (Randox Laboratories. Ltd., UK). After 15 weeks, mice were anaesthetized with pentobarbital (150mg/kg i.p.). The aortic sinus and thoracic aorta were gently removed and the adipose tissue peeled off the aortic wall. Subsequently, the aortic sinus and thoracic aorta were fixed with 4% paraformaldehyde solution before cryosection.

SSAO activity analysis

SSAO activity was determined as a result of the turnover of hydrogen peroxide,

which was measured by Amplex Red Monoamine Oxidase Assay Kit (A12214, Molecular Probes, Eugene, OR, USA). Briefly, 100μg of protein was incubated at room temperature with clorgyline 1 μM (monoamine oxidase-A inhibitor), pargyline 3 μM (monoamine oxidase-B inhibitor), benzylamine 2 mM (substrate of SSAO), 100 μM Amplex Red reagent, and 1 U/ml HRP, with or without semicarbazide 1mM. Absorbance at 570 nm was measured every 5 minutes for 30 minutes. A standard curve was plotted using different solutions with known hydrogen peroxide concentrations. Production rate of hydrogen peroxide is calculated and expressed as $[H_2O_2]$/min/μg protein. SSAO activity was determined by the difference between the production rates of hydrogen peroxide with and without semicarbazide. The linearity (R2) of this assay is 0.9989~1.0000 and the intra- or inter-assay CV is 0.5~3.8% in our lab.

Oil Red O staining

Atherosclerotic plaque development was quantified by histomorphometry of Oil Red O-stained aortic sinus and thoracic aorta. Aortic sinus and thoracic aorta stained with Oil Red O were performed according to a standard protocol. Briefly, after being rinsed with water, the aorta was exposed to isopropanol (60%) for 2 min, followed by 1 h of staining with a solution of Oil Red O dissolved in 60% isopropanol. The excess stain was removed with isopropanol and water, and the aorta was photographed using

a microscope equipped with a digital camera (Leica). The Oil Red-stained areas representing atherosclerotic plaques were measured using Image Pro Plus. The results were expressed as the ratio of total plaque area over total vessel area. The aortic sinus and thoracic aorta were fixed in 4% paraformaldehyde, embedded in OCT, and cross-sectioned for morphometric analysis and immunohistochemistry. The aortic sinus and thoracic aorta were cut serially into 8 μm frozen sections, and every tenth section was stained with Oil Red O for lesion area calculation. The analyses were performed microscopically; the images were analyzed with Image Pro Plus.

Evaluation of atherosclerotic lesions and immunohistochemical staining

For the pathomorphological examination, 8 μm-thick serial sections were collected throughout the length of each segment. All sections were immunohistochemically stained with CD31 (1:200, endothelial marker, Abcam, Cambridge, UK), α-actin (1:200, SMC marker, GeneTex, Irvine, California), Iba-1 (1:500, macrophage marker, Wako, Richmond, VA), monocyte chemoattractant protein-1 (MCP-1) (1:100, Pepotech, Rocky. Hill, NJ), Toll-like receptor-4 (TLR-4) (1:100, Proteintech, Chicago, IL, USA), CD36 (1:100, Abcam, Cambridge, UK), receptor for advanced glycation end-products (RAGE) (1:100, Abbiotec, San Diego, CA), matrix metalloproteinase-9 (MMP-9), VAP-1, vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), E-selectin, proliferating cell

nuclear antigen (PCNA) (1:100, all from Santa Cruz, Delaware Avenue, CA), tumor necrosis factor alpha (TNF-α), lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) (1:100, all from GeneTex, Irvine, California), nitrotyrosine (1:100, upstate Biotechnology, Charlottesville, VA) and then reacted with rhodamine- and FITC-conjugated secondary antibodies (1:200; Jackson ImmunoResearch, USA), and observed by fluorescence microscopy.

Western blot detection

The cells were lysed with RIPA lysis buffer (Cell Signaling, Beverly, MA, USA). A total of 20 μg of protein was separated by 10-12% SDS–PAGE and transferred onto PVDF membranes (Millipore, Bedford, MA, USA). After blocking with 5% BSA at room temperature for 1 h, the membranes were incubated with primary antibodies (all 1:1000 in 1.5% BSA in TBST) at 4°C overnight. The membranes were then incubated with HRP-conjugated secondary antibodies (1:6000 in 1.5% BSA in TBST) at room temperature for 1 h. Immunoreactivity was detected with ECL (GE Healthcare Bioscience, Piscataway, NJ, USA).The intensities of the bands were quantified using Gel-Pro software (Media Cybernetics, Rockville, MD, USA). β-actin or GAPDH was used as an internal control (1:6000 in 1.5% BSA in TBST; GeneTex, Irvine, California). The intensities of the target proteins were normalized by the intensities of the internal control bands.

Tissue adhesion assay

The cultured U937 cells were stained with the BCECF-AM. The stained U937 cells were added to the artery for 30 min at 37 °C. They were then washed and observed by fluorescence microscopy.

Cell cultures

A7r5, purchased as cryopreserved tertiary cultures (Cascade Biologics Inc., Portland, OR, USA), were grown in DMEM containing 10% FBS and 1% antibiotic/antimycotic solution at 37°C in a 5% CO_2 atmosphere. The growth medium was changed every other day until confluence, and then the cells were passaged every 3-5 days. Cells between passages 3 and 8 were used for the subsequent experiments. Before conducting experiments, A7r5 were pre-cultured in serum-starved medium for 24 h. Human umbilical vein endothelial cells (HUVEC) were obtained from the Bioresource Collection and Research Center (BCRC). The cells were grown in Endothelial Cell Medium (ECM) (Lonza, Walkersville, MD, USA) containing penicillin-streptomycin (1%) and endothelial cell growth supplements at 37°C in a humidified atmosphere of 95% air and 5% CO_2 and were used between passages 2 and 5. HUVEC overexpressing VAP-1/SSAO (HUVEC hSSAO/VAP-1 cells) and A7r5 cells overexpressing VAP-1/SSAO (A7r5 hSSAO/VAP-1 cells) were developed by Dr. Mercedes Unzeta and Dr. Montse Solé (Universitat Autònoma de Barcelona (UAB), Barcelona, Spain). The details were described in previous

publications.[44,45] HUVEC hSSAO/VAP-1 and A7r5 hSSAO/VAP-1 cells were cultured similarly ways as described for HUVEC and A7r5 cells. U937 cells were obtained from ATCC (American Type Culture Collection). Cells were grown in RPMI-1640 medium (GIBCO) supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100 Ag/ml streptomycin at 37℃ in an incubator containing 5% CO2.

ROS detection

Cells were grown in 6 cm culture dishes. After the cells were pre-treated with 300 nM PXS-4728A for 1 h, and 1 μg/mL of lipopolysaccharide (LPS) or 10 ng/mL TNF-α for another 1 h, the cells were stained with dichlorofluorescein diacetate (H_2DCF-DA) and then were trypsinized for FACScan cytometer analysis.

Monocyte adhesion assay

HUVEC hSSAO/VAP-1 cells were grown in 24-well culture plates. After reaching confluence, cells were pre-treated with 300 nM PXS-4728A or N-acetyl-L-cysteine (NAC) (5, 10 mM) for 1 h, and 10 ng/mL TNF-α treatment for another 24 h. After treatment, the U937 cells were added to treated cells for 60 min at 37 °C. They were then washed and observed under an inverted phase-contrast microscope.

Monocyte transmigration assay

HUVEC hSSAO/VAP-1 cells were grown in transwells. After reaching

confluence, cells were pre-treated with 300 nM PXS-4728A for 1 h, and 10 ng/mL TNF-α treatment for another 24 h. After treatment, the U937 cells were added into transwells for another 6 h. The transmigrated cells were determined after 6 h by Coomassie blue staining and count.

Cell cycle analysis

A7r5 hSSAO/VAP-1 cells were grown in 6-well culture plates and then serum-starved as described above. After pretreatment with 300 nM PXS-4728A or NAC (1, 2 mM) for 1 h and 20 ng/mL platelet-derived growth factor (PDGF)-BB for another 24 h, the cells were trypsinized and stained with propidium iodide (PI). The cell-cycle progression was detected on a FACScan cytometer (BD Biosciences, San Jose, CA) and was analyzed by ModFit software.

Wound-healing assay

A7r5 hSSAO/VAP-1 cells were grown in 6 cm culture dishes. After the cells were pre-treated with 300 nM PXS-4728A or NAC (1, 2 mM) for 1 h, wounds were inflicted by dragging a sterile pipette tip across the monolayer, creating a 250 μm cell-free path after which 20 ng/mL PDGF-BB (Peprotech, Rocky Hill, NJ, USA) was added. After 24 h, the migration rate of the cells and the wound closure area/original wound area ratio were calculated using a real-time cultured cell monitoring system (ASTEC, Japan) and Metamorph and Image-Pro Plus 4.5 software.

Statistical analyses

Continuous variables with normal distribution were presented as means ± SD or means ± SEM. Variables with skewed distribution were presented as medians (interquartile ranges) and were analyzed after logarithmic transformation. The statistical significance of the differences in different subgroups was tested with Student's *t* test or χ^2 test. Unadjusted Pearson's correlation coefficients and partial correlation coefficients adjusting for age and gender were used to determine the relationship of plasma VAP-1/SSAO with cardiovascular risk factors, the number of vessels with stenosis and the number of segments with stenosis. The association between CAD and plasma VAP-1/SSAO was evaluated by logistic regression; whereas the association between the number of vessels with stenosis, the number of segments with stenosis, and plasma VAP-1/SSAO was evaluated by linear regression analyses. Clinically important variables associated with CAD were included in multivariate analyses, including age, gender, hypertension, diabetes, smoking and LDL-C. A two-tailed *p*-value below 0.05 was considered significant. Stata/SE 14.0 for Windows (StataCorp LP, College Station, TX) was used for statistical analyses.

Results

Expression of VAP-1/SSAO in atherosclerotic lesions in human and ApoE-deficient

mice

To examine VAP-1/SSAO expression during atherosclerosis in humans and mice, immunohistochemical staining was performed with antibodies against VAP-1/SSAO. In the human normal coronary artery, little VAP-1/SSAO expression was seen (Figure 1A). Compared to normal artery, VAP-1/SSAO expression was significantly stronger in the atherosclerotic plaques. To examine the cellular localization of VAP-1/SSAO during the formation of atherosclerosis in humans and ApoE-deficient mice, immunohistochemical staining with antibodies against VAP-1/SSAO, endothelial cells, or SMC was carried out in human coronary artery and the mice aorta sections (Figure 1A and 1B). VAP-1/SSAO staining overlaid with markers staining for endothelial cells and SMC (Figure 1A and 1B), thus confirming that endothelial cells and SMC in atherosclerotic plaques express VAP-1/SSAO. In Supplemental Figure 1, VAP-1/SSAO expression in thoracic aorta increased by time after cholesterol-enriched diet was given. However, there was no significant difference in plasma VAP-1/SSAO concentrations at different time after cholesterol-enriched diet was given (means ± standard deviations, at day 0, 1.007 ± 0.7418 ng/ml; at the 9^{th} week, 1.972 ± 1.726 ng/ml; at the 12^{th} week, 3.276 ± 2.330 ng/ml; at the 15^{th} week, 1.639 ± 0.613 ng/ml, $p>0.05$).

Plasma VAP-1/SSAO concentrations are positively associated with the presence and the extent of CAD in humans

Baseline characteristics of the subjects enrolled in the human study are shown in Table 1. A total of 127 (70%) subjects were diagnosed with CAD. Compared to those without CAD, subjects with CAD were more likely to be male, had a higher prevalence to use statins, and had lower plasma HDL-C. There were no significant differences between the two groups regarding age, smoking, body mass index (BMI), blood pressure, history of hypertension or diabetes, plasma concentrations of fasting and 2-h postprandial glucose, hemoglobin A1c, total cholesterol, triglyceride, and LDL-C. Notably, plasma VAP-1/SSAO concentrations were significantly higher in subjects with CAD than in the control group (median 579 [interquartile range 490-710] ng/ml vs. 544 [460-644] ng/ml, p=0.003, Table 1 and Figure 2A). There was a positive association between plasma VAP-1/SSAO concentrations, the number of coronary arteries with stenosis (p for trend<0.001, Figure 2B), and the number of coronary arterial segments with stenosis (p for trend=0.001, Figure 2C).

Table 2 showed the relationships between plasma VAP-1/SSAO concentrations and clinical characteristics. In univariate analysis, plasma VAP-1/SSAO positively correlated with age (r=0.1485, p=0.047), fasting plasma glucose (r=0.2349, p=0.0015), 2-h postprandial plasma glucose (r=0.3435, p<0.0001), the number of

coronary arteries with stenosis (r=0.2569, p=0.0005), and the number of coronary arterial segments with stenosis (r=0.2505, p=0.0007). The relationships remained significant after controlling for age and gender. There was a significant correlation between plasma VAP-1/SSAO and plasma triglyceride concentrations (r=0.1731, p=0.021), adjusted for age and gender. Besides, plasma VAP-1/SSAO concentrations were similar between subjects receiving statins and subjects who did not receive statins (median 570 [interquartile range 475-674] ng/ml vs. 578 [489-652] ng/ml, p=0.99).

To determine whether plasma VAP-1/SSAO was an independent predictor of the presence and the extent of CAD, we performed logistic and linear regression analyses (Table 3). Plasma VAP-1/SSAO concentrations were associated with the presence of CAD (odds ratio=2.09, 95% CI 1.29-3.38, p=0.003, adjusted for age and gender). There was a positive association between plasma VAP-1/SSAO concentrations and the extent of CAD, as measured by the number of coronary arteries with stenosis (β=0.32, p<0.001) and the number of coronary arterial segments with stenosis (β=0.62, p<0.001). The relationship of plasma VAP-1/SSAO concentrations to the presence and the extent of CAD remained significant after further adjustment for history of hypertension, diabetes, plasma LDL-C and smoking.

PXS-4728A inhibits VAP-1/SSAO activity and the consequent H_2O_2 generation and oxidative stress in cholesterol-fed ApoE-deficient mice

To detect the effects of PXS-4728A treatment on VAP-1/SSAO activity, we measured SSAO-specific H_2O_2 production rate in cholesterol-fed ApoE-deficient mice.PXS-4728A significantly inhibited VAP-1/SSAO activity and SSAO-specific H_2O_2 production in thoracic aortic tissues, lung, and epididymal fat (Figure 3A-C). The oxidative stress expression was determined by nitrotyrosine staining. The result showed that the oxidative stress expression was decreased significantly by PXS-4728A treatment (Figure 3D).

SSAO inhibition by PXS-4728A reduces atherosclerosis in cholesterol-fed ApoE-deficient mice

The effects of PXS-4728A on the atherosclerotic plaque area were quantified by histomorphometric analysis of the aortic sinus and thoracic aortic sections in ApoE-deficient mice 15 weeks after cholesterol-enriched diet. The atherosclerotic plaque area was determined by Oil Red O staining analysis (Figure 3E-F). The ratios of atherosclerotic plaque area to total area of the aortic sinus and thoracic aorta were significantly higher in the cholesterol-fed group than in the control group. Compared with the cholesterol-fed group, PXS-4728A significantly reduced the ratio of

atherosclerotic plaque area to total area, both in the prevention group and in the treatment group (51.69% ± 2.27% in the cholesterol-fed group; 36.05% ± 3.93% in the prevention group [PXS-4728A given along with cholesterol-enriched diet for 15 weeks], $p<0.05$ vs. cholesterol-fed group; 36.65% ± 4.29% in the treatment group [cholesterol-enriched diet was given for 8 weeks followed by cholesterol-enriched diet plus PXS-4728A for another 7 weeks] , $p<0.05$ vs. cholesterol-fed group). Similar data were observed in the ratios of atherosclerotic plaque area to total area of thoracic aorta. Importantly, the effect of VAP-1/SSAO inhibition in the prevention group was similar to the effect of atorvastatin (2.5 mg/kg/day for 15 weeks), the treatment of choice for primary and secondary prevention for cardiovascular diseases in humans.

Effects of VAP-1/SSAO inhibition by PXS-4728A on serum biochemical parameters of cholesterol-fed ApoE-deficient mice

As shown in Table 4, plasma total cholesterol, LDL-C, triglyceride and glucose concentrations were significantly increased in the cholesterol-fed group, compared with the control group ($p<0.05$). The elevation of plasma total cholesterol, LDL-C, and glucose concentrations were significantly reduced by PXS-4728A (the prevention group) or atorvastatin (all $p<0.05$). In the PXS-4728A 7W group (the treatment group), plasma glucose concentrations decreased significantly, but plasma total cholesterol

and LDL-C were not significantly different from that in the cholesterol-fed group. In all groups, there was no significant difference in plasma creatinine, AST and ALT.

SSAO inhibition by PXS-4728A reduces the expression of adhesion molecules and inflammatory cytokines in atherosclerotic plaques

To test the effects of PXS-4728A on inflammation, immunohistochemical staining and western blot with antibodies against adhesion molecules and inflammatory cytokines were carried out on thoracic aorta sections. The expression of adhesion molecules, including VAP-1, vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) and E-selectin, were higher in thoracic aorta of ApoE-deficient mice in the cholesterol-fed group than that in the control group (Figure 4A and Supplemental Figure 2). VAP-1/SSAO inhibition by PXS-4728A (both the prevention and the treatment group) significantly decreased the expression of these adhesion molecules, compared with the cholesterol-fed group (Figure 4A and Supplemental Figure 2).

Since leukocyte recruitment plays an important role in the initiation and progression of atherosclerosis,[4,5] we used labeled-U937 cells to directly test whether VAP-1/SSAO inhibition can inhibit early monocyte recruitment into atherosclerotic lesions. In Figure 4B-C, the number of labeled-U937 cells on artery was higher in the

cholesterol-fed group than that in the control group (Figure 4B-C). PXS-4728A (both in the prevention and the treatment groups) significantly decreased the number of monocytes adhered to the arteries, compared with the number in the cholesterol-fed group. In Figure 4D and Supplemental Figure 2, the expression of pro-inflammatory molecules TNF-α and MCP-1 were higher in the cholesterol-fed group and were reduced by PXS-4728A (both in the prevention and the treatment groups). All together, the results suggest that, VAP-1/SSAO inhibition by PXS-4728A reduce inflammation and leukocyte recruitment to atherosclerotic lesions in cholesterol-fed ApoE-deficient mice.

SSAO inhibition by PXS-4728A reduces the expression of markers for macrophage recruitment and activation in atherosclerotic plaques

TLR-4, CD36 and LOX-1 activation by oxidized LDL (oxLDL) contribute to the pathogenesis of atherosclerosis.[46] During the development of atherosclerosis, macrophages are recruited to plaques, where they are activated by AGEs and oxLDL.[47] Activated macrophages form foam cells, which consequently propagate inflammation and progresses to atherosclerosis.[48,49] To test the effect of VAP-1/SSAO inhibition by PXS-4728A on AGEs-LDL-activated macrophages in the atherosclerotic plaques, immunohistochemical staining and western blot with antibodies against Iba-1

(a macrophage marker), TLR-4, CD36, RAGE and LOX-1 were performed in ApoE-deficient mice thoracic aorta sections. As shown in Figure 4E and Supplemental Figure 2, the cholesterol-enriched diet resulted in a significant increase in the recruitment of macrophages and the expression of TLR-4, CD36, RAGE and LOX-1 in the atherosclerotic plaques. These effects were markedly decreased by PXS-4728A, except CD36 and LOX-1 expression in the cholesterol-fed/PXS7W group. All together, these data showed that VAP-1/SSAO inhibition by PXS-4728A reduces the expression of markers for macrophage recruitment and activation in atherosclerotic plaques.

SSAO inhibition by PXS-4728A reduces the adhesion and the transmigration of monocytes in TNF-α-treated HUVEC overexpressing hSSAO/VAP-1 by inhibiting ROS production

In order to elucidate the role of VAP-1/SSAO in endothelial cells on atherosclerosis, HUVEC hSSAO/VAP-1 cells were used. In Figure 5A-B, HUVEC hSSAO/VAP-1 cells expressed more VAP-1/SSAO protein than HUVEC, as determined by western blot and immunofluorescence analysis. The VAP-1/SSAO activity of HUVEC hSSAO/VAP-1 cells was inhibited completely by PXS-4728A treatment (Figure 5C).

Since TNF-α-induced endothelial dysfunction and ROS production play a pivotal role in the pathogenesis of atherosclerosis,[50] the effect of VAP-1/SSAO inhibition was also tested in the presence of TNF-α stimulation. As shown in Figure 5D, the accumulation of intracellular ROS was increased by TNF-α treatment, and the increased effect was significantly inhibited by PXS-4728A treatment, suggesting that VAP-1/SSAO inhibition can reduce TNF-α-induced ROS production. During inflammation, monocytes are adhered in the vascular wall and then transmigrate into subendothelial space.[51] Therefore, we evaluated the effect of VAP-1/SSAO inhibition on the adhesion and transmigration of monocytes. As shown in Figure 5E and F, the number of adhered and transmigrated monocytes were increased by TNF-α treatment. PXS-4728A pre-treatment significantly reduced the number of monocytes adhered to and transmigrated across TNF-α-treated HUVEC hSSAO/VAP-1 cells. Besides, pretreatment with NAC, a ROS scavenger, also reduced the number of adhered and transmigrated monocytes. Taken together, these findings suggest that VAP-1/SSAO inhibition by PXS-4728A reduced the adhesion and transmigration of monocytes in TNF-α-treated HUVEC hSSAO/VAP-1 cells by inhibiting the production of ROS.

SSAO inhibition by PXS-4728A reduces the expression of MMP-9, SMC, and PCNA in atherosclerotic plaques

The proliferation and migration of vascular SMC play an important role in the progression of atherosclerosis. SMC migrate from the tunica media into the intima via degradation of the extracellular matrix mediated by MMP-9 and other proteinases.[52,53] In Figure 4F and Supplemental Figure 2, analysis of MMP-9 and the SMC marker, α-actin, revealed stronger MMP-9 expression and more α-actin-positive cells in the thickened intima of the cholesterol-fed group as compared to the intima of the control group. These effects were significantly reduced by PXS-4728A treatment. To detect cell proliferation in the atherosclerotic plaque, PCNA staining was used. As shown in Figure 4F and Supplemental Figure 2, the expression and the number of PCNA-positive cells in the thickened plaques were higher in the cholesterol-fed group than that in the control group and in PXS-4728A groups. Taken together, these findings suggest that VAP-1/SSAO inhibition by PXS-4728A reduces the expression of markers for SMC migration and proliferation in the development of atherosclerosis.

SSAO inhibition by PXS-4728A reduces the cell proliferation and migration in A7r5 cells overexpressing hSSAO/VAP-1 by inhibiting ROS production

In order to elucidate the role of VAP-1/SSAO in SMC on atherosclerosis, A7r5 hSSAO/VAP-1 cells were used. In Figure 6A-C, A7r5 hSSAO/VAP-1 cells expressed more VAP-1/SSAO protein than A7r5 cells, and had VAP-1/SSAO activity. Treatment

with PXS-4728A inhibited the VAP-1/SSAO activity of A7r5 hSSAO/VAP-1 cells completely (Figure 6C).

ROS have been reported to accelerate the proliferation and migration of vascular SMC.[54] As shown in Figure 6D, LPS treatment led to intracellular ROS accumulation in A7r5 hSSAO/VAP-1 cells, which was significantly reduced when cells were treated with PXS-4728A. The proliferation and migration of vascular SMC are important in the progression of atherosclerosis.[55] First, we measured the effects of PXS-4728A on SMC proliferation by flow cytometry. As shown in Figure 6E, following PDGF-BB stimulation, the proportion of A7r5 hSSAO/VAP-1 cells in S phase increased from 7.72 ± 0.43% to 12.79 ± 0.65% ($p<0.05$), which was prevented by PXS-4728A pre-treatment (12.79 ± 0.65% vs. 8.45 ± 0.25%, $p<0.05$) and accompanied by a significant increase in the proportion of cells in G0/G1 phase (from 73.33 ± 2.19% to 78.05 ± 1.33%, $p<0.05$). These data indicate that PXS-4728A suppresses the proliferation of PDGF-BB-treated A7r5 hSSAO/VAP-1 cells via G0/G1 arrest. Besides, pre-treatment with ROS scavenger NAC also reduced PDGF-BB-stimulated proliferation of A7r5 hSSAO/VAP-1 cells. For SMC migration, wound-healing assays were done. As shown in Figure 6F, the migration of A7r5 hSSAO/VAP-1 cells increased significantly after stimulation with PDGF-BB. PXS-4728A or NAC treatment reduced migration rate of PDGF-BB–treated A7r5 hSSAO/VAP-1 cells

significantly. Taken together, these findings indicate that VAP-1/SSAO inhibition by PXS-4728A reduces the proliferation and migration of PDGF-BB–treated A7r5 hSSAO/VAP-1 cells by inhibiting ROS production.

Discussion

In the present study, we have demonstrated that plasma VAP-1/SSAO concentrations were higher in subjects with CAD and were positively associated with the number of coronary arteries or coronary arterial segments with stenosis in humans. The findings are different from the results in a study by Salmi *et al.*,[37] who did not found any significant association between serum VAP-1/SSAO and CAD. Since the diagnosis of CAD in the study was established by self-reporting and verified with drug reimbursement records, but not by coronary angiography as in the present study, the possible ascertainment bias may reduce the statistical power. Aside from CAD, there are several articles which report a significant relationship between serum VAP-1/SSAO and atherosclerosis in carotid arteries in humans. Serum VAP-1/SSAO activity has been reported to be associated with carotid intima-medial thickness and plaques in women[22] and in diabetic subjects.[36] In general population, we have demonstrated that acute change of serum VAP-1/SSAO to hyperglycemia was correlated to blood AGEs concentrations and carotid intima-medial thickness.[23]

Furthermore, we have reported that serum VAP-1/SSAO can predict cardiovascular mortality and all-cause mortality in subjects with diabetes;[25] whereas in subjects older than 50 years, a Finnish study has shown that serum VAP-1/SSAO can predict the incidence of major cardiovascular events and cardiovascular mortality.[24] All these results suggest that blood VAP-1/SSAO is a biomarker for atherosclerosis and can be used to predict cardiovascular events and mortality.

A recent study supports our findings that endothelial cells in atherosclerotic plaques displayed increased VAP-1 expression compared to normal artery, and VAP-1/SSAO inhibition reduced the macrophage recruitment in atherosclerotic plaques.[28] In LDL receptor-deficient mice expressing only apolipoprotein B100 ($LDLR^{-/-}ApoB^{100/100}$), VAP-1 was expressed on the luminal surface of endothelial cells in atherosclerotic plaques. However, endothelial cells lining non-atherosclerotic vessel wall were VAP-1 negative. When a small molecular VAP-1 inhibitor (LJP1586) was given after introducing high-fat diet for 8 weeks, it reduced the density of macrophages in plaques. In contrast to our study, there was no significant difference in the size of plaques after LJP1586 treatment for 4 weeks. This discrepancy may be explained by a shorter treatment length (4 weeks), which may not be long enough to observe the significant regression of atherosclerosis. Besides, mice were shifted to a normal chow diet during LJP1586 treatment, which may slow down the progression

of atherosclerosis. Therefore, the effect of LJP1586 on atherosclerosis was minimized, so that no significant difference in the size of plaques was found between the treatment and control groups.

RAGE is a member of immunoglobulin superfamily that is able to bind diverse molecules such as AGEs, ALEs and high mobility group box 1 (HMGB1), and function as a signaling transduction receptor.[56] The interaction of RAGE and its ligands can induce the release of inflammatory cytokines, increase expressions of adhesion molecules, and promote SMC proliferation, thereby contributing to atherogenesis.[57] RAGE inactivation has been shown to inhibit atherosclerosis through reducing oxLDL-induced pro-inflammatory responses and oxidative stress in LDL receptor-deficient mice.[58] In the present study, VAP-1/SSAO inhibition decreased H_2O_2 production and the expression of RAGE in the vessel walls, suggesting two possible mechanisms whereby VAP-1/SSAO inhibition reduces atherosclerosis.

In the present study, plasma VAP-1/SSAO concentrations correlated positively with plasma fasting glucose and 2-h postprandial glucose in humans (Table 2), and VAP-1/SSAO inhibition by PXS-4728A significantly lowered fasting plasma glucose in ApoE-deficient mice (Table 4). In the literature, serum VAP-1/SSAO activity has been reported to correlate with plasma glucose concentrations in humans.[20,21,37] Besides, a population study found an association of serum VAP-1/SSAO activity with

type 1 diabetes in both sexes and with type 2 diabetes in men.[22] There are two possible mechanisms linking VAP-1/SSAO and glucose homeostasis. First, vascular VAP-1/SSAO participates in inflammation of adipose tissue. Adipose macrophages can secrete various cytokines, such as TNFα, IL-1β and IFNγ, which results in insulin resistance in adipose tissue and promotes lipolysis.[59] Increased circulating fatty acids may lead to ectopic lipid accumulation and insulin resistance in liver and skeletal muscle through the generation of diacylglyceride and protein kinase C. In VAP-1/SSAO knockout mice, leukocyte infiltration in adipose tissue was reduced.[60] Besides, these mice had an increased fat mass, suggesting that their fat storage capacity was increased and may be more resistant to insulin resistance resulting from ectopic lipid accumulation. In contrast, adipose VAP-1/SSAO may play a beneficial role by enhancing glucose uptake induced by amines, which has been shown in the experiments using fat cells from VAP-1/SSAO knockout mice[44] and in KKAy mice using another SSAO inhibitor (E)-2-(4-fluorophenethyl)-3-fluoroallylamine (FPFA)[61]. In KKAy mice, a single injection of FPFA resulted in elevation of plasma glucose. However, chronic treatment of FPFA on glucose homeostasis was not evaluated in the study. Taken together, since inhibition of adipose inflammation may take a longer time to develop, we hypothesize that VAP-1/SSAO inhibition may increase plasma glucose concentrations through the inhibition of adipose glucose uptake in a short period of

time, but may improve glucose homeostasis through the reduction of adipose inflammation in a long period of time, as shown in the present study. Further studies are needed to prove the hypothesis in the future.

Hypercholesterolemia is an important risk factor for atherosclerosis. In humans, serum VAP-1/SSAO activity has been reported to correlate with plasma LDL-C[22] and total cholesterol.[62] In the present study, VAP-1/SSAO inhibition by PXS-4728A for 15 weeks significantly reduced plasma LDL-C levels in cholesterol-fed apolipoprotein E-deficient mice. However, plasma LDL-C levels was not significantly suppressed with PXS-4728A for 7 weeks, suggesting that treatment duration is an important factor for plasma LDL-C reduction. Indeed, inhibition of VAP-1/SSAO by another specific inhibitor LJP1586 for 4 weeks failed to show any change in plasma LDL-C in $LDLR^{-/-}$ $ApoB^{100/100}$ mice.[28] The mechanism for the effect remains unknown. Several pro-inflammatory cytokines and stimulants, such as TNFα, IL-1 and lipopolysaccharide, have been reported to increase serum cholesterol levels by enhancing hepatic cholesterol synthesis and 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase activity, and reducing bile acid synthesis in rodent models.[63] However, in humans, subjects with inflammatory diseases, such as rheumatoid arthritis and psoriasis, have lower levels of plasma LDL-C, which can be elevated by anti-inflammatory agents.[64,65] In these patients, there are changes in quality of LDL-C,

such as generation of small dense LDLs and increased susceptibility toward oxidation etc.[63] Therefore, they have an increased risk of atherosclerosis despite a lower plasma LDL-C levels. Further studies are needed to clarity the mechanisms how VAP-1/SSAO inhibition reduces plasma LDL-C, considering the treatment duration and differences in research models. Clinically, statins are the treatment of choice for primary and secondary prevention of cardiovascular events, mainly through the reduction in plasma LDL-C.[66,67] However, concerns about the association between statins and new-onset diabetes have recently been raised. In the present study, VAP-1/SSAO inhibition has shown to successfully reduce plaque size, and the efficacy of VAP-1/SSAO inhibition for primary prevention of atherosclerosis is comparable to that of atorvastatin. Since PXS-4728A for 7 weeks reduced plaque size without a significant decrease in plasma LDL-C levels, this suggests that VAP-1/SSAO inhibition can alleviate the progression of atherosclerosis through not only lipid-dependent mechanisms but also lipid-independent mechanisms, such as decreasing ROS production, lowering plasma glucose, suppressing endothelial activation, inhibiting recruitment and activation of macrophages, and attenuating migration and proliferation of SMC. Taken together, our findings suggest that VAP-1/SSAO inhibition is a novel way with different mechanisms to statins for the treatment of atherosclerosis.

The first step to initiate atherosclerosis is the activation of endothelial cells, which facilitates leukocyte trafficking to inflammatory sites, thereby kicks off the downstream pathological immune response. Therefore, the role of VAP-1/SSAO on endothelial cells serves as a crucial cornerstone for the anti-atherosclerotic effects of PXS-4728A. VAP-1/SSAO on endothelial cells not only serves as an adhesion molecule[6] but also acts as an ectoenzyme contributing to leukocyte rolling, firm adhesion and transmigration[68-71]. A working model has been proposed to explain how VAP-1/SSAO modulates the leukocyte extravasation cascade, based on study results using anti-VAP-1 antibodies and VAP-1/SSAO inhibitors and HUVEC transfected with an enzyme-inactive mutant of VAP-1.[10] First, VAP-1 on endothelial cells serves as an adhesin to interact with leukocytes through antibody-defined epitopes. Then, the leukocyte surface molecule is used as a substrate for oxidative deamination reaction of SSAO. This leads to the formation of a transient covalent interaction (Schiff base) which brings endothelial cells and leukocytes together. Finally, after the enzyme reaction proceeds, the leukocyte surface molecule is catalyzed to aldehyde and H_2O_2, which can serve as a signaling molecule to regulate leukocyte emigration and modify inflammatory cascades in the local microenvironment. In the present study, VAP-1/SSAO inhibition reduced monocyte adhesion to aorta (Figure 4B and 4C), as well as adhesion to and transmigration through HUVEC hSSAO/VAP-1 monolayers

(Figure 5E and Figure 5F). Our results indicate that abrogation of VAP-1/SSAO activity inhibits a sequential process associated with endothelial activation. First, VAP-1/SSAO inhibition reduced the expressions of E-selectin, P-selectin, and ICAM-1 on aorta (Figure 4A and Supplemental Figure 2), which supports the concepts that the catalytic activity of VAP-1/SSAO can up-regulate several other endothelial adhesion molecules.[72,73] Notably, we found that inhibition of VAP-1/SSAO activity also reduced its own protein expression on endothelial cells (Figure 4A and Supplemental Figure 2). This finding implies that there might be a positive feedback mechanism by which VAP-1/SSAO activity could regulate its own protein expression, but further studies are warranted to prove this hypothesis. Moreover, we found that VAP-1/SSAO inhibition suppressed intracellular ROS production in HUVEC hSSAO/VAP-1 cells (Figure 5D). In the literature, intracellular ROS have been shown to regulate the expression of adhesion molecules.[74,75] In addition, we have proven that the number of adhered and transmigrated monocytes was decreased by antioxidant NAC (Figure 5E and 5F). Taken together, our results suggest that VAP-1/SSAO inhibition can reduce monocyte adhesion and transmigration, which may result from direct enzymatic effect, suppressed intracellular ROS production and reduced expression of adhesion molecules.

During inflammation, chemotactic proteins such as MCP-1 are released to recruit

leukocytes, especially monocytes, into the inflammatory site.[4] Once monocytes have migrated into the intima, they can be stimulated by various cytokines and differentiate into macrophages. In the present study, cholesterol-enriched diet resulted in the upregulation of the expression of MCP-1 and various pro-inflammatory cytokines, and more macrophages were recruited in the atherosclerotic plaques. By contrast, VAP-1/SSAO inhibition reduced the expression of MCP-1 and these cytokines, and suppressed macrophage recruitment. These findings are in agreement with previous reports.[73,76] In a rat model, VAP-1/SSAO inhibition has been shown to suppress the expression of MCP-1 and TNF-α, resulting in reduced choroidal neovascularization.[76] Similarly, decreased expression of MCP-1 and TNF-α by VAP-1/SSAO inhibition has also been demonstrated in a mice model of intracerebral hemorrhagic stroke.[73]

Proliferation and migration of vascular SMC are crucial to atherogenesis.[55] In the present study, SMC displayed strong expression of VAP-1/SSAO, which is also found in a previous report.[77] During differentiation of SMC, the mRNA expression, protein expression, and VAP-1/SSAO activity increase significantly.[78] Mercier *et al*. have demonstrated that VAP-1/SSAO deficient mice had larger arterial diameters, suggesting a role of VAP-1/SSAO in arterial wall remodeling.[79] MMP-9, with its proteolytic activity, is a key molecule in SMC migration and vessel remodeling.[52,53] In the literature, intracellular ROS can also regulate the migratory and proliferative

activities of SMC, aside from various inflammatory cytokines and growth factors.[80-82] ROS participate in pro-migratory signaling pathways, such as lamellipodia formation, actin cytoskeleton remodeling, focal adhesion turnover, and contraction of cell body.[81] ROS also promote SMC proliferation and modulate the activity and gene expression of MMP.[82] In the present study, VAP-1/SSAO inhibition decreased MMP-9 expression in aorta (Figure 4F and Supplemental Figure 2), reduced ROS production in A7r5 hSSAO/VAP-1 SMC (Figure 6D), and attenuated the migratory and proliferative ability of PDGF-BB-treated A7r5 hSSAO/VAP-1 SMC (Figure 6E and 6F). Moreover, we have demonstrated that inhibition of oxidative stress by NAC suppressed the proliferation and migration of SMC (Figure 6E and 6F). Taken together, our findings indicate that VAP-1/SSAO inhibition can regulate proliferation and migration of SMC, directly or indirectly via its effect on MMP-9 expression and intracellular redox state.

In the present study, we found that VAP-1/SSAO inhibition by PXS-4728A reduced atherosclerosis. However, the findings are different from the two reported studies, both used semicarbazide to inhibit VAP-1/SSAO in LDL receptor knock-out mice on western-type diet.[26,27] When semicarbazide was given after introducing western-type diet for 6 or 9 weeks, it increased collagen content and cap thickness of the plaques, and induced phenotypic switch of SMC, but did not result in regression of the atherosclerotic lesions.[26] By contrast, when semicarbazide was given along with

western-type diet, it resulted in an increase in the size of atherosclerotic lesions.[27] One of the potential reasons for the discrepant findings is the off-target effects of semicarbazide. Semicarbazide is not a specific inhibitor for VAP-1/SSAO and can act on other enzymes involved in atherogenesis.[29,30] For instance, semicarbazide can inhibit LOX, another amine oxidase involved in extracellular matrix maturation and proliferation, as well as SMC migration. The disturbance of LOX expression could contribute to endothelial dysfunction and plaque progression.[31] In addition, semicarbazide can also inhibit S1P lyase,[29] a key regulator of S1P signaling. S1P has diverse effects on a variety of cell types in atherogenesis, including attachment and migration of monocytes, SMC proliferation, and production of pro-inflammatory cytokines.[32,33] Thus, disturbance of S1P signaling may have pro- or anti-atherogenic effects, depend on the cell and animal model studied.[34] By contrast, we used a potent and highly specific VAP-1/SSAO inhibitor PXS-4728A, which has more than 500-fold selectivity for VAP-1/SSAO over all the related human amine oxidases (including LOX) or over 100 different macromolecular targets.[35] Therefore, off-target effects are minimized in this study.

The present study is limited in that the human study measured plasma VAP-1/SSAO protein concentrations alone, rather than plasma VAP-1/SSAO protein concentrations and VAP-1/SSAO activity. However, serum VAP-1/SSAO protein

concentrations have been shown to correlate well with serum VAP-1/SSAO activity,[22,37] and treatment with antibody against VAP-1 can reduce more than 95% of serum VAP-1/SSAO activity.[37] Therefore, it is reasonable to speculate that subjects with CAD in this study not only have elevated plasma VAP-1/SSAO protein concentrations but also have higher plasma VAP-1/SSAO activity. Besides, there was a marked male predominance in subjects with CAD, which may result from chance since we recruited study subjects consecutively and did not select them by gender. Since the relationship between plasma VAP-1 and the presence or the extent of CAD was similar after adjustment for gender, this limitation has limited effect on our findings.

In conclusion, plasma VAP-1/SSAO concentrations are associated with CAD in humans, and can be used as a novel biomarker for assessing the presence and the extent of CAD. PXS-4728A, a specific VAP-1/SSAO inhibitor, can reduce atherosclerosis in cholesterol-fed ApoE-deficient mice, through suppression of many key steps for atherosclerosis, including ROS generation, endothelial dysfunction, adhesion and transmigration of monocytes, recruitment and activation of macrophages, as well as migration and proliferation of SMC. Our data suggest that VAP-1/SSAO inhibition is a potential treatment for atherosclerotic cardiovascular disease.

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Conflicts of Interest: All authors have read the journal's authorship agreement and policy on disclosure of potential conflicts of interest, and have none to declare.

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Figure legends

Figure 1. Expression of vascular adhesion protein-1 (VAP-1) is increased in atherosclerotic plagues and is colocalized with endothelial cells (EC) and smooth muscle cells (SMC). Immunohistochemical staining with the antibodies against EC, SMC, and VAP-1 by double immunofluorescent staining in (A) coronary artery sections in humans (n = 3-4) and in (B) aorta sections in apolipoprotein E (ApoE)-deficient mice (n = 3-4). VAP-1 staining (white arrows) is stronger in atherosclerotic plaques than that in nomal parts in human aorta. VAP-1 staining is colocalized with EC and SMC. The scale bar = 100 μm in (A) and 50 μm in (B). Nuclei are stained with DAPI. Dashed white line represents internal elastic lamina.

Figure 2. Plasma vascular adhesion protein-1 (VAP-1) is positively associated with the presence and extent of coronary arterial disease (CAD) in humans. (A) Subjects with CAD (n = 127) had higher plasma VAP-1 concentrations compared with those without CAD (n = 53). (B) Plasma VAP-1 concentrations was positively associated with the number of coronary arteries with clinical significant stenosis (n = 53, 38, 39, and 50, respectively). (C) Plasma VAP-1 concentrations was positively associated with the number of coronary arterial segments with clinical significant stenosis (n = 53, 26, 18, 25, 21, 18, and 19, respectively). Means and standard errors (error bars) of log-transformed (Ln) plasma VAP-1 are shown.

Figure 3. PXS-4728A inhibits semicarbazide-sensitive amine oxidase (SSAO) activity and reduces cholesterol diet-induced atherosclerosis in apolipoprotein E (ApoE)-deficient mice. Effects of PXS-4728A on SSAO activity in (A) thoracic aorta, (B) lung, and (C) epididymal fat. SSAO activity is expressed as the SSAO-specific production rate of H_2O_2 (n = 4-8). (D) Representative images and quantification of the intensity of nitrotyrosine staining within the thoracic aorta. The values are the mean ± SEM (n = 3-5 per group). The scale bar = 50 μm. (E) Oil Red O staining of aortic sinus and quantification of the Oil Red O positive area (n = 5-8). The scale bar = 100 μm. (F) En face Oil Red O staining of thoracic aorta, and quantification of the Oil Red O positive area represented as percentage of total aorta area (n = 6-9). The values are the mean ± SEM. *p <0.05. (CTRL, control group; cholesterol-fed, cholesterol-enriched diet for 15 weeks; cholesterol-fed/PXS, PXS-4728A (10 mg/kg/day) was given along with cholesterol-enriched diet for 15 weeks, "the prevention group"; cholesterol-fed/PXS 7W, cholesterol-enriched diet was given for 8 weeks followed by cholesterol-enriched diet plus PXS-4728A (10 mg/kg/day) for another 7 weeks, "the treatment group"; cholesterol-fed/atorvastatin, atorvastatin (2.5 mg/kg/day) was given along with cholesterol-enriched diet for 15 weeks, " the positive therapeutic control group".)

Figure 4. Semicarbazide-sensitive amine oxidase (SSAO) inhibition by PXS-4728A suppresses expression of adhesion molecules, inflammatory cytokines, markers for macrophage recruitment and activation, as well as markers for smooth muscle cells (SMC) migration and proliferation in apolipoprotein E (ApoE)-deficient mice. (A) Expression of adhesion molecules including vascular adhesion protein-1(VAP-1), vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and E-selectin in the atherosclerotic plaques by immunohistochemical staining in different groups. The negative control containing only a rhodamine-conjugated secondary antibody without primary antibody incubation (w/o 1° primary antibody) showed the species specificity of the antibody. (B) En face microscopy images illustrate BCECFAM-labeled U937 bound to endothelium of thoracic aorta. (C) Quantification of the number of monocytes adhered to aorta in different groups, represented as the percentage of control. The values are the mean ± SEM (n = 3-5 per group). *$p<0.05$ (D) Expression of inflammatory cytokines including monocyte chemoattractant protein-1 (MCP-1) and tumor necrosis factor-α (TNF-α) in the atherosclerotic plaques by immunohistochemical staining in different groups. (E) Expression of markers for macrophage recruitment and activation including Iba-1 (macrophage), Toll-like receptor-4 (TLR-4), CD36, receptor for advanced glycation end-product (RAGE), and lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) in the atherosclerotic plaques by immunohistochemical staining in different groups. (F) Expression of matrix

metalloproteinase-9 (MMP-9), smooth muscle cell marker α-actin (SMC), and proliferative cell nuclear antigen (PCNA) in the atherosclerotic plaques by immunohistochemical staining in different groups. The internal elastic lamina is indicated by the arrows. The negative control containing only a rhodamine- or FITC-conjugated secondary antibody without primary antibody incubation (w/o 1° primary antibody) showed the species specificity of the antibody. The scale bar = 100 μm. (CTRL, control group; cholesterol-fed, cholesterol-enriched diet for 15 weeks; cholesterol-fed/PXS, PXS-4728A was given along with cholesterol-enriched diet for 15 weeks; cholesterol-fed/PXS 7W, cholesterol-enriched diet was given for 8 weeks followed by cholesterol-enriched diet plus PXS-4728A for another 7 weeks.)

Figure 5. Semicarbazide-sensitive amine oxidase (SSAO) inhibition by PXS-4728A reduces reactive oxygen species (ROS) production and monocyte transmigration in tumor necrosis factor-α (TNF-α)-induced human umbilical vein endothelial cells (HUVEC) overexpressing SSAO/VAP-1 (HUVEC hSSAO/VAP-1 cells). (A-C) vascular adhesion protein-1 (VAP-1) expression and SSAO activity were determined by western blot, immunofluorescence and SSAO activity assay in HUVEC and HUVEC hSSAO/VAP-1 cells. (D) Cells were pre-treated with PXS-4728A (300 nM) for 1h and then treated with TNF-α (10 ng/ml) for another 1h. After treatment, the cells were then loaded with ROS indicator

dichlorofluorescein diacetate (H_2DCF-DA), and the fluorescence intensity was assessed by flow cytometry and quantified. (E) SMC were pretreated with PXS-4728A (300 nM) or N-acetyl-L-cysteine (NAC) (5, 10 mM) for 1 h and then treated with 10 ng/ml TNF-α for another 24 h. The monocyte adhesion assay was performed to evaluate dysfunction of TNF-α-treated HUVEC hSSAO/VAP-1 cells. (F) Cells were pre-treated with PXS-4728A (300 nM) or NAC (5, 10 mM) for 1h and then treated with TNF-α (10 ng/ml) for another 24 h. After treatment, monocytes were added in the upper well. Monocytes transmigrated for 6 h onto the lower surface of the filter were stained with coomassive blue and counted. The bar graphs represent the numbers of cells transmigrated. The values are the mean ± SEM (n = 3-6 per group). *p <0.05. The scale bar = 100 μm. CTRL, control; PXS, PXS-4728A.

Figure 6. Semicarbazide-sensitive amine oxidase (SSAO) inhibition by PXS-4728A reduced reactive oxygen species (ROS) production, proliferation and migration in lipopolysaccharide (LPS)- or platelet-derived growth factor (PDGF)-BB-induced A7r5 cells overexpressing SSAO/VAP-1 (A7r5 hSSAO/VAP-1 cells). (A-C) vascular adhesion protein-1 (VAP-1) expression and SSAO activity were determined by western blot, immunofluorescence and SSAO activity assay in A7r5 smooth muscle cells andA7r5 hSSAO/VAP-1 cells. (D) Cells were pre-treated with PXS-4728A (300 nM) for 1h and then treated with LPS (1 μg/ml) for another 1h. After treatment, the cells were loaded with ROS

indicator dichlorofluorescein diacetate (H_2DCF-DA), and fluorescence intensity was assessed by flow cytometry and quantified. (E) Cells were left untreated or were treated with 300 nM PXS-4728A or N-acetyl-L-cysteine (NAC) (1, 2 mM) for 1h and then with PDGF-BB for additional 24 h. The proportion of cells in the S phase was determined by FACScan analysis. (F) Cell migration was examined via wound-healing assay. The migration rate of the cells was determined. The values are the mean ± SEM (n = 3-5 per group). *p <0.05. The scale bar = 100 μm. CTRL, control; PXS, PXS-4728A.

Table 1. Clinical characteristics of total population stratified by the presence of coronary arterial disease (CAD).

Variable	CAD (-)	CAD (+)	p
N	53	127	
Numbers of coronary arteries with stenosis (0/1/2/3, N)	**53/0/0/0**	**0/38/39/50**	**<0.001**
Numbers of coronary arterial segments with stenosis	**0**	**3.5 ± 2.0**	**<0.0001**
Age (years)	57.9 ± 12.2	61.5 ± 11.6	0.066
Gender (male/female)	**31/22**	**109/18**	**<0.001**
Smoking (no/yes/quit)	29/11/13	59/27/41	0.5
BMI (kg/m^2)	25.6 ± 3.9	26.5 ± 4.1	0.15
Systolic blood pressure (mmHg)	135 ± 19	140 ± 23	0.3
Diastolic blood pressure (mmHg)	74 ± 9	76 ± 12	0.2
Use of anti-hypertensive drugs (N, %)	34 (64)	80 (63)	0.9
Hypertension (N, %)	46 (87)	115 (91)	0.5
Fasting plasma glucose (mg/dL)	96 ± 19	108 ± 61	0.2
2-h postprandial plasma glucose (mg/dL)	139 ± 59	157 ± 86	0.2
Hemoglobin A1c (%)	6.3	6.2	0.7
Diabetes (N, %)	22 (42)	51 (40)	0.9
Total cholesterol (mg/dL)	178 ± 30	170 ± 35	0.11
Triglyceride (mg/dL)	110 (79-151)	116 (86-179)	0.072
LDL-C (mg/dL)	99 ± 25	94 ± 32	0.3
HDL-C (mg/dL)	**44 ± 11**	**40 ± 12**	**0.03**
Use of statin medications (N, %)	**7 (13)**	**58 (46)**	**<0.001**
Plasma VAP-1 (ng/ml)	**544 (460-644)**	**579 (490-710)**	**0.003**

Means ± SDs or medians (interquartile ranges) are shown.

Abbreviations: BMI, body mass index; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; VAP-1, vascular adhesion protein-1

Table 2. The relationship between plasma vascular adhesion protein-1 (VAP-1) and clinical characteristics. Pearson's correlation coefficients (*r*) and partial correlation coefficients (partial *r*) are shown. Plasma VAP-1 and triglyceride levels were log transformed for the analysis.

Variable	*r*	p	partial *r**	p*
Age (years)	**0.1485**	**0.047**		
BMI (kg/m^2)	0.0246	0.7	0.0794	0.30
Systolic blood pressure (mmHg)	0.1063	0.16	0.0871	0.25
Diastolic blood pressure (mmHg)	-0.0307	0.68	0.0015	0.98
Fasting plasma glucose (mg/dL)	**0.2349**	**0.0015**	**0.2524**	**0.0007**
2-h postprandial plasma glucose (mg/dL)	**0.3435**	**<0.0001**	**0.3687**	**<0.0001**
Hemoglobin A1c (%)	0.0897	0.3	0.0866	0.3
Total cholesterol (mg/dL)	-0.0479	0.5	-0.0593	0.43
Triglyceride (mg/dL)	0.1426	0.056	**0.1731**	**0.021**
LDL-C (mg/dL)	-0.1224	0.10	-0.1324	0.081
HDL-C (mg/dL)	-0.0597	0.4	-0.1150	0.13
The number of coronary arteries with stenosis	**0.2569**	**0.0005**	**0.2869**	**0.0001**
The number of coronary arterial segments with stenosis	**0.2505**	**0.0007**	**0.2717**	**0.0003**

* Adjust for age and gender

Abbreviations: BMI, body mass index; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol

Table 3. The relationship between plasma vascular adhesion protein-1 (VAP-1) and the presence or the extent of coronary arterial disease (CAD) in adjusted models. Odds ratios (OR) or regression coefficients (β) for every 1 standard deviation increase in plasma VAP-1 are shown.

	The presence of CAD		The number of coronary arteries with stenosis		The number of coronary arterial segments with stenosis	
	OR (95% CI)	p	β	p	B	p
Crude	**1.81 (1.19-2.76)**	**0.006**	**0.30**	**<0.001**	**0.61**	**<0.001**
Model 1	**2.09 (1.29-3.38)**	**0.003**	**0.32**	**<0.001**	**0.62**	**<0.001**
Model 2	**2.27 (1.36-3.77)**	**0.002**	**0.34**	**<0.001**	**0.66**	**<0.001**

Model 1, adjusted for age and gender.

Model 2, adjusted for age, gender, hypertension, diabetes, plasma low-density lipoprotein cholesterol and smoking.

Table 4. The clinical and biochemical characteristics of apolipoprotein E-deficient mice.

	CTRL	Cholesterol-fed	Cholesterol-fed/ PXS	Cholesterol-fed/ PXS 7W	Cholesterol-fed/ atorvastatin
Body weight (Day 0)	27.0 ± 0.6	27.8 ± 0.5	26.3 ± 0.5	26.3 ± 0.4	25.7 ± 0.4
Body weight (Day 105)	30.2 ± 0.7	**40.7 ± 1.8***	**40.3 ± 2.1***	**42.3 ± 1.1***	**38.1 ± 1.3***
Fasting plasma glucose (mg/dl)	129 ± 8	**197 ± 15***	**156 ± 11†**	**157 ± 7†**	**139 ± 11†**
Total cholesterol (mg/dl)	281 ± 21	**464 ± 26***	**374 ± 35†**	**436 ± 33***	**384 ± 24†**
Triglyceride (mg/dl)	116 ± 8	**190 ± 13***	**188 ± 15***	**191 ± 12***	**201 ± 24***
LDL-C (mg/dl)	234 ± 20	**415 ± 25***	**324 ± 37†**	**383 ± 33***	**338 ± 24†**
HDL-C (mg/dl)	47 ± 2	48 ± 2	51 ± 6	53 ± 6	46 ± 2
Creatinine (mg/dl))	0.50 ± 0.10	0.46 ± 0.05	0.49 ± 0.07	0.63 ± 0.09	0.67 ± 0.11
AST (U/L)	21 ± 2	29 ± 2	29 ± 2	30 ± 2	29 ± 3
ALT (U/L)	21 ± 5	30 ± 3	34 ± 6	34 ± 4	39 ± 6

Means ± SEM are shown.

*p <0.05 compared with control group. †p <0.05 compared with Cholesterol-fed group.

Abbreviations: LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; aspartate aminotransferase, AST; alanine transaminase, ALT

CTRL, control group; Cholesterol-fed, cholesterol-enriched diet for 15 weeks; cholesterol-fed/PXS, PXS-4728A was given along with cholesterol-enriched diet for 15 weeks; cholesterol-fed/PXS 7W, cholesterol-enriched diet was given for 8 weeks followed by cholesterol-enriched diet plus PXS-4728A for another 7 weeks; cholesterol-fed/atorvastatin, atorvastatin was given along with cholesterol-enriched diet for 15 weeks.

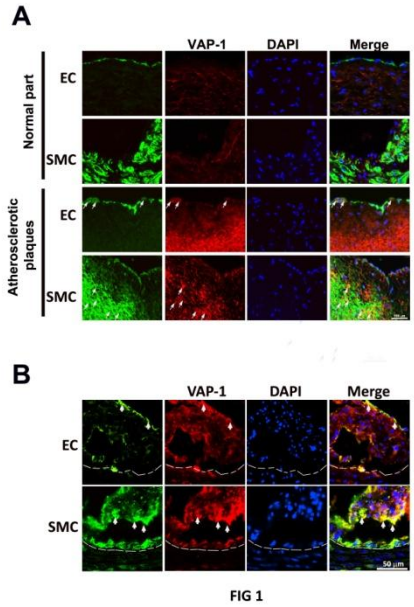

FIG 1

1

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3

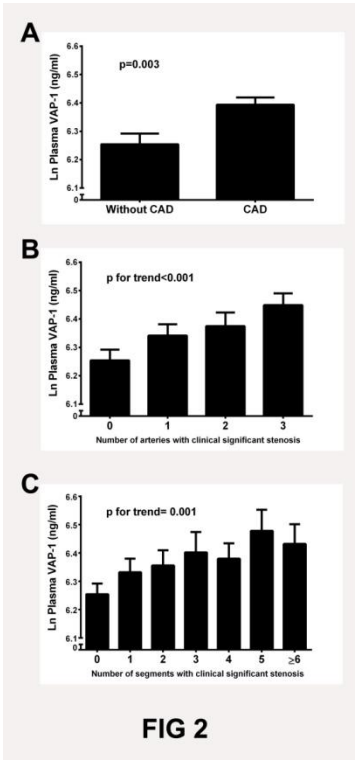

Fig 2 HUMAN.tif

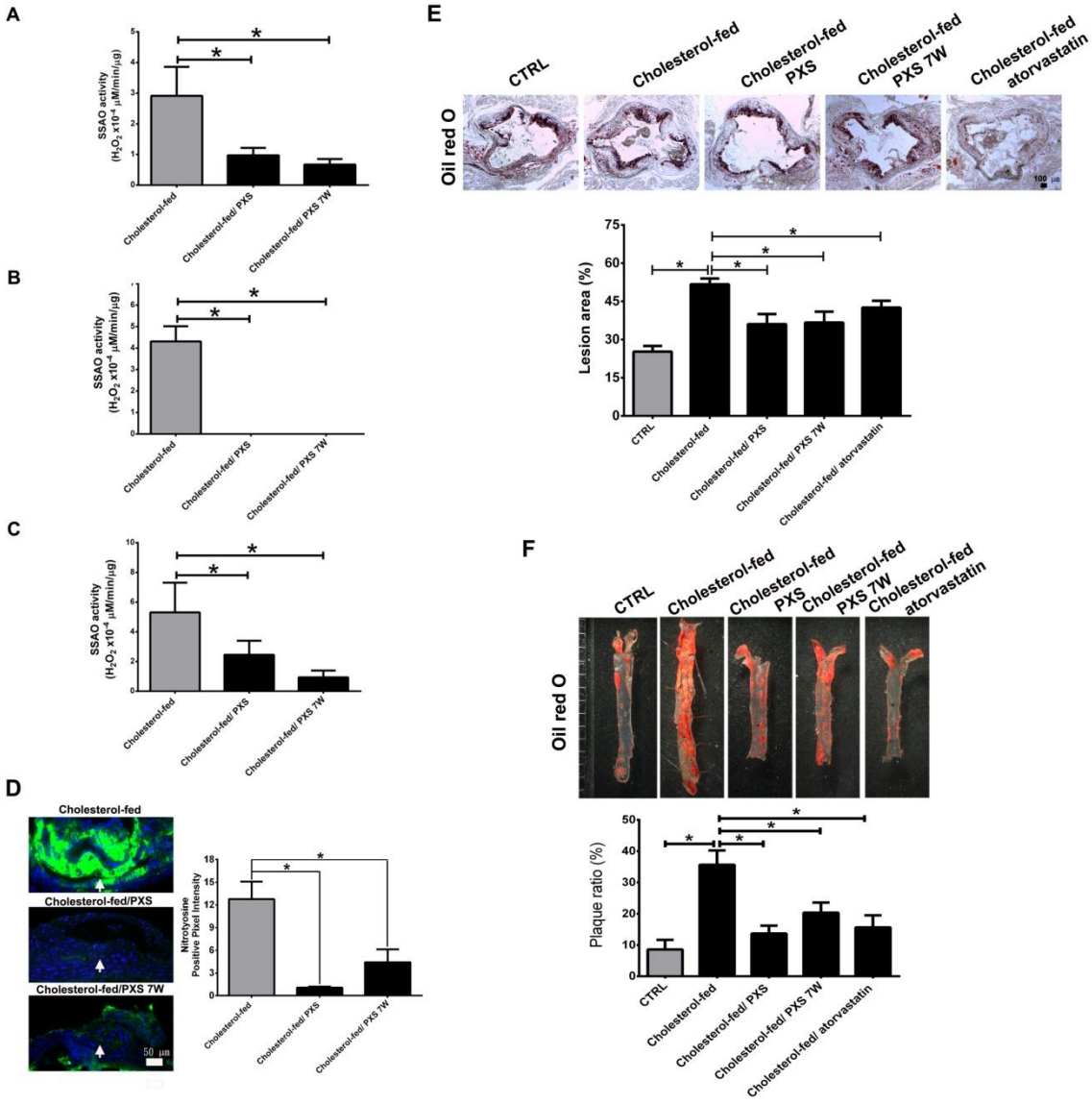

FIG 3

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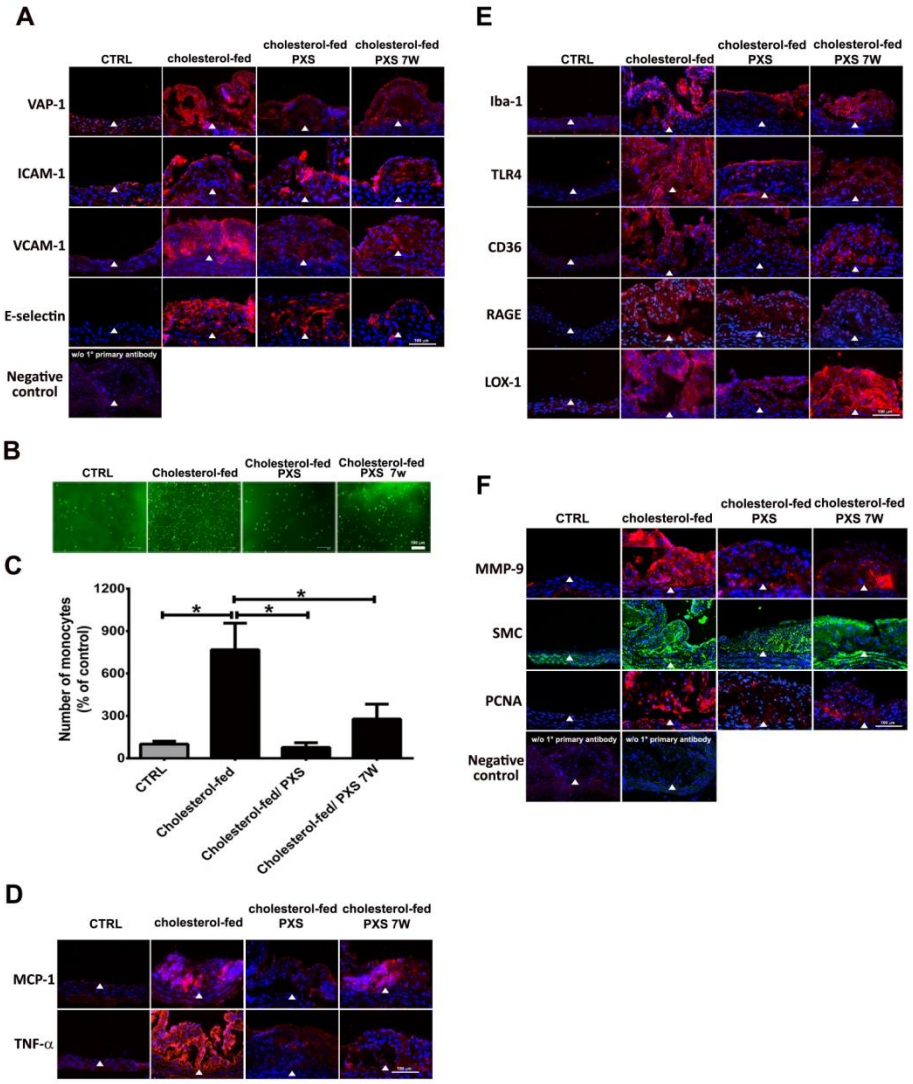

FIG 4

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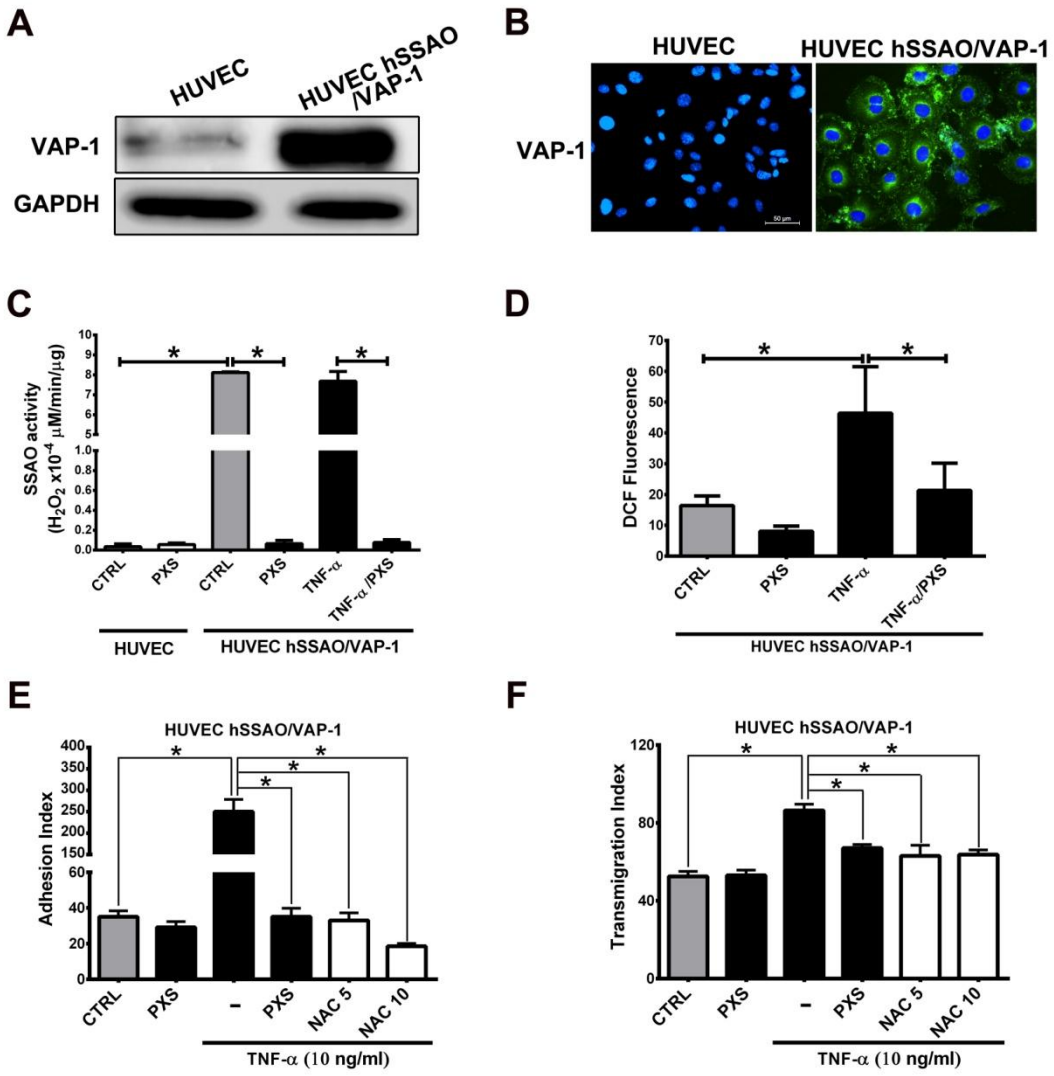

FIG 5

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2

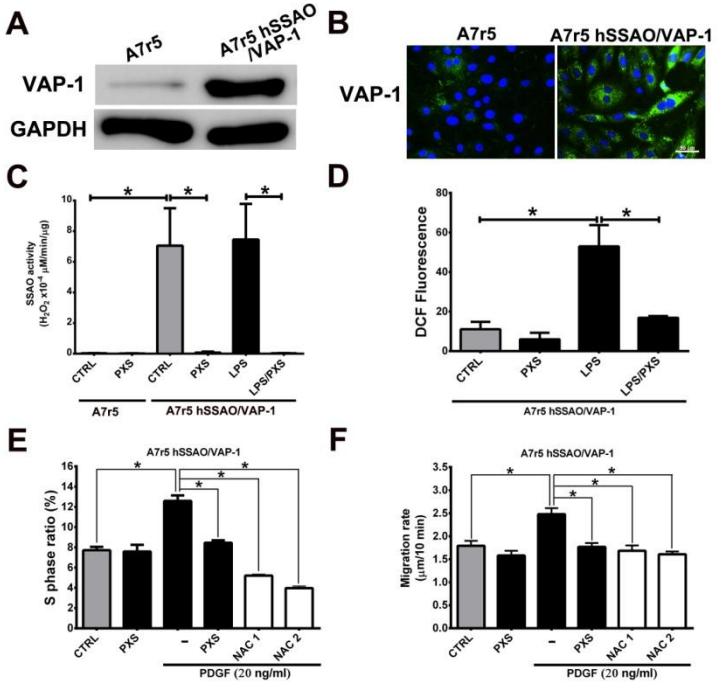

FIG 6

1

2 Fig 6 A7r5 20180214.tif